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SHORT NOTES

Isolation and Characterization of 17 Microsatellite DNA Loci for *Odorrana margaretae* (Anura: Ranidae)

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Abstract We isolated and characterized 17 microsatellite DNA loci for the odorous frog *Odorrana margaretae* from its transcriptome sequence data. These loci were screened with 24 individuals from Mt. Emei. All loci were polymorphic, with the number of alleles ranging from 2 to 8. The observed and expected heterozygosity, polymorphism information content, ranged from 0.04 to 1, 0.04 to 0.81, and 0.040 to 0.763, respectively. All loci were in linkage equilibrium and six loci were significantly deviated from Hardy−Weinberg equilibrium after sequential Bonferroni corrections. Cross−species amplification test was conducted for ten odorous frog species, and 12 loci were amplifiable in most species. With the high cross−species amplification rates, these markers will provide useful molecular tools for conservation genetic and phylogeographic studies on the genus *Odorrana* and *Bamburana*.

Keywords Microsatellite DNA loci, Transcriptome, *Odorrana margaretae*, Cross−species amplification test

The green odorous frog *Odorrana margaretae* is widely distributed in mountain regions of southwestern China. It is a dominant amphibian species in the forest−stream ecosystem, and plays an important role in the function and stability of this ecosystem (Frost, 2014). In addition, the species, as well as most other odorous frogs, are reservoirs for antimicrobial peptides (AMPs) and have recently attracted much attention for the property (Yang *et al.*, 2012).

Microsatellite DNA markers are useful tools for understanding population genetic structure and mating system, as well as for parentage analysis and genetic resource assessment due to their high polymorphism, relatively small size, and well−established analysis protocols (Ellegren, 2004; Jones *et al.*, 2010; Mittal and Dubey, 2009). A major obstacle in developing microsatellite DNA markers, however, is its tedious isolating processes (Guichoux *et al.*, 2011). Recently, with the advances of next generation sequencing technologies, transcriptome sequence data offer an alternative fast, inexpensive, and accurate way to identify microsatellite DNA loci in non−model organisms (Guichoux *et al.*, 2011). We recently reported a transcriptome profile of *O. margaretae* (Qiao *et al.*, 2013), and subsequently we designed and tested potential useful microsatellite DNA markers for this species based on its trancriptome data. In this paper, we report 17 polymorphic microsatellite DNA loci for *O. margaretae* and their cross−species amplifiability for ten closely−related species.

Qiao *et al.* (2013) identified 2,574 microsatellite DNA loci from the *O. margaretae* transcriptome sequence data and designed 11,695 pairs of primers using the QDD2 pipeline (Meglecz *et al.*, 2010). In this study, we selected 60 pairs of primers based on their parameter values to test for amplifiability and polymorphism. A total of 35 pairs of primers were successfully amplified for *O. margaretae*. PCR amplifications were performed in 15 μl reaction volume containing 0.6 μl of genomic DNA, 0.6 μl of each primer, 5.7 μl ddH_2O, and 7.5 μl 2 × *EasyTaq* SuperMix (TransGen Biotech). The amplification conditions were as following: an initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing at temperature T_a for 30 s (Table 1), and extension at 72°C for 30 s; and a final extension at 72°C for 10 min.

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PCR products were visualized on 8% nondenaturing polyacrylamide gels with silver staining, and their polymorphism was assessed. Ultimately, 17 loci were readily amplifiable and polymorphic, and we proceeded to characterize these loci.

A total of 24 individuals of *O. margaretae* were collected from Emei Mountain, China (E 103°38918' , N 29°56418' , 749 m) in June 2012, and used to characterize the 17 microsatellite DNA loci. The forward primers were labeled with a fluorescent dye (FAM, HEX or TAMRA). The sizes of the PCR products were determined on an ABI3730xl DNA Analyzer (Applied Biosystems) and analyzed using GeneMarker®HID v1.95 analysis software. Each locus was amplified separately and loci with different fluorescent dyes were pooled for size determination. Hardy-Weinberg equilibrium (HWE), linkage disequilibrium (LD), and population genetic parameters were analyzed using Genepop on the web version 4.2 (Raymond and Rousset, 1995). Polymorphism information content (*PIC*) was calculated by Cervus 3.0.7 (Kalinowski *et al.*, 2007). A sequential Bonferroni correction was applied for multiple tests (Rice, 1989). The null alleles and possible scoring errors were assessed by Micro-Checker 2.2.3 (Van Oosterhout *et al.*, 2004).

As shown in Table 1, the numbers of alleles (N_A) and *PIC* for each locus varied from 2 to 8 (average 4.1) and from 0.040 to 0.763, respectively. The observed heterozygosities (H_o) ranged from 0.04 to 1, and the expected heterozygosities (H_e) ranged from 0.04 to 0.81. Six loci (Om10, Om14, Om21, Om25, Om52, Om57) showed significantly deviation from HWE ($P < 0.01$) after the Bonferroni correction, and the micro−checker analysis showed no evidence for scoring error or technical or statistical artifacts. Significant deviation from LD ($P < 0.01$) was not observed. The high number of loci deviated from HWE is likely a consequence of the Wahlund effect, because these 24 individuals were collected from multiple streams in close vicinity, which may not represent a true random mating population.

We tested ten closely−related species for cross−species amplifiability, including *Bamburana nasuta*, *O. kuangwuensis*, *O. wuchuanensis*, *O. graminea*, *O. schmackeri*, *O. hejiangensis*, *O. junlianensis*, *O.grahami*, *O. nanjiangensis*, and *O. lungshengensis*. Two individuals of each species were tested. High success rates of cross−amplification were obtained, and eight pairs of primers were able to amplify all ten species, and 4 pairs were able to amplify 8 or 9 species (Table 1).

The transcriptome data and next generation sequencing techniques prove to be a much better alternative than conventional methods for isolating microsatellite DNA loci. It is much less time consuming and relatively inexpensive. Furthermore, the primers tested in this study are amplifiable across a large number of species, which will provide useful molecular tools for conservation genetic and phylogeographic studies of odorous frog species.

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Table 1 Characterization of 17 microsatellite markers for *Odorrana margaratae*, and cross−amplification among one *Bamburana* and nine *Odorrana* species tested with 2 samples each. Species abbreviations: 'Bn'= *Bamburana nasuta*, 'Ok'= *O. kuangwuensis*, 'Ow'= *O. wuchuanensis*, 'Og1'= *O. graminea*, 'Os'= *O. schmackeri*, 'Oh'= *O. hejiangensis*, 'Oj'= *O. junlianensis*, 'Og2'= *O. grahami*, 'On'= *O. nanjiangensis*, and 'Ol'= *O. lungshengensis*.

Locus	Primer sequence (5'−3') (F, forward; R, reverse)	Repeat motif	T_a (°C)	*Odorrana margaratae* (*n* = 24)						Cross−amplification (*n* = 2)									
				N_A	Size Range (bp)	*P*	H_o	H_e	*PIC*	Bn	Ok	Ow	Og1	Os	Oh	Oj	Og2	On	Ol
Om10	F: TGCCTCCTTCCTTCCATACA R: ACATACACATCGGGCCAATC	(ACCT)5	63	2	272−276	—	0.04	0.04	0.040	×	√	√	×	×	×	×	×	×	×
Om14	F: TGAAGAGGAGGATGAGGCAG R: CACCCTCCCAATCTCTTTCC	(AAG)15	60	4	143−158	0.0082	0.29	0.56	0.457	√	√	√	√	√	√	√	√	√	√
Om16	F: AGCTCGCTATGTCGGATGAC R: TTGGAGAGGTCCCGGTCTAT	(AGG)11	60	2	094−097	0.1970	0.58	0.45	0.346	√	√	√	√	√	√	√	√	√	√
Om17	F: ACGACAGAGCTGCCAGAGAC R: GTCCGCTTTCATTCTGCACT	(ACT)10	60	5	094−106	0.0131	0.79	0.69	0.623	√	√	√	√	√	√	√	√	√	√
Om21	F: GGCGGGACAGAATCCATAAC R: ACCTGCTTGGAGAAGCTGTG	(AGG)8	56	6	102−129	0.0000	0.33	0.74	0.679	√	√	√	√	√	√	√	√	√	√
Om25	F: TGAATGATGCCACTTGATCG R: CCGGACGTGAAGTCCATAAC	(AG)17	56	6	219−269	0.0000	0.13	0.55	0.721	×	×	×	×	×	×	×	×	×	×
Om29	F: ACATTGGAAGATCCAGGGTG R: AGCTGGAACTGAAGAAATGCT	(AG)13	64	2	104−106	1.0000	0.21	0.19	0.169	√	√	√	√	√	√	√	√	√	√
Om32	F: AAGTGTGTTGGTCAGGCGTT R: CATTTGAACTTTGAATGTGTGTTG	(AGAT)8	60	7	115−151	0.7745	0.75	0.81	0.763	×	√	√	×	×	×	×	×	×	×
Om37	F: ATTTGTTCATCCCAGCAAGG R: AAAGGACCTCTACACAAATGTTGAT	(AAT)7	59	2	153−159	0.2047	0.13	0.19	0.169	√	√	√	√	√	√	√	√	√	√
Om38	F: GTCGGCGTGTTCCTGTAAGT R: TCATTGCGGTCCATATCTCC	(AGG)7	60	2	145−160	1.0000	0.25	0.22	0.195	√	√	√	×	√	√	√	√	√	√
Om40	F: AGCAAATTTGTCAAGCCCTG R: CCAGCTTGCTGTCCTGGTAG	(AAT)7	63	6	145−166	0.0259	1.00	0.72	0.661	×	√	√	×	√	√	√	√	√	√
Om46	F: GACAGGAAGCAGTGGGAATG R: TCCCATCCAGGTGTAACAGG	(AAT)7	56	4	160−187	0.5035	0.79	0.70	0.623	×	√	×	×	×	×	√	√	×	√
Om50	F: CACTGCGACAAGTCATCAGC R: CTCATGCCATCCTTGGGTAG	(ACG)6	60	2	146−149	1.0000	0.13	0.12	0.110	√	√	√	×	√	√	√	√	√	√
Om52	F: CATGTCTTCCAGCCCGGTAT R: ATGAGGATTATCGGTGTCCG	(AAT)6	40	5	123−135	0.8094	0.67	0.57	0.519	√	√	√	√	√	√	√	√	√	√
Om53	F: TGGGTATTGCTGGCTCTTTG R: CTAATGCCAGACCCAACAGG	(AAT)6	53	4	211−232	0.0014	0.46	0.70	0.621	×	√	√	√	√	√	√	√	√	√
Om54	F: GGCCCACGTGTACAACAAAT R: AAGCCTTGGCTACAGACTCG	(AAT)6	53	8	255−279	0.1290	0.83	0.70	0.659	√	√	√	√	√	√	√	√	√	√
Om57	F: TTGAATCCCTCAGTTTACCTGC R: CCTGGAAGCCAACAATTCAG	(ACT)6	53	3	170−176	0.0000	1.00	0.59	0.459	×	√	√	×	×	×	×	×	×	×

T_a = optimal annealing temperature, NA= number of alleles, bp = base pairs, *P* = value from exact tests for Hardy-Weinberg equilibrium, H_o = observed heterozygosity, H_e = expected heterozygosity, *PIC* = polymorphism information content, — = no information, × = failed to amplify, √ = succeed to amplify.